Modified Oxygen Radical Absorbance Capacity Assay that can be Implemented at Low Temperatures: A pilot study

Shunji KOHRI^{*†} and Hirotada FUJII

Center for Medical Education, Sapporo Medical University, South-1 West-17, Chuo-ku, Sapporo, Hokkaido 060-8556, Japan

Received November 5, 2012; Accepted December 10, 2012

In this paper, the feasibility of a modified oxygen radical absorbance capacity assay based on fluorescence (ORAC-FL) was evaluated. In the modified ORAC-FL assay, a large quantity of radicals is generated in a short period of time by photolysis of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), through illumination with ultraviolet light. Importantly, no heating is required to generate the radicals, while the time required to monitor the fluorescence intensity (FI) is shortened, owing to the rapid fluorescence decay. In this study, the optimal experimental conditions for the modified assay were determined and the antioxidant capacity of two samples was evaluated at room temperature using the modified assay. Although relative ORAC values were obtained, some aspects of the modified assay require further optimization for eventual practical use.

Keywords: oxygen radical absorbance capacity, ORAC-FL, low temperature, antioxidant activity

Introduction

Chronic diseases such as heart diseases, strokes and cancer, as well as chronic respiratory diseases and diabetes, are the leading cause of mortality worldwide (i). Experimental evidence that oxidative stress is involved in several chronic diseases has been accumulating (Fernández-Sánchez *et al.*, 2011; Ha *et al.*, 2010; Lonkar *et al.*, 2011). In addition to direct treatment, prevention plays an important role in combating these conditions. An important strategy for prevention could be the removal of reactive oxygen species to reduce oxidative stress. Fruits and vegetables contain antioxidants that remove reactive oxygen species (Guo *et al.*, 1997; Sommerburg *et al.*, 1998), and there is increasing interest in the reduction of the oxidative stress through daily diet, i.e., by consumption of fruits and vegetables (Lordan *et al.*, 2011; Traka *et al.*, 2011). From this point of view, it is meaningful to evaluate the oxygen-radical scavenging activity of food samples, as it will allow the selection of fruits and vegetables with high oxygen radical scavenging ability, and the development of processed foods with high scavenging ability,

thereby allowing more efficient intake of antioxidants.

Several methods that determine oxygen radical scavenging activity have been developed to date (Huang *et al.*, 2005; Karadag *et al.*, 2009). Among these methods, the fluorescence-based oxygen radical absorbance capacity (ORAC-FL) assay is a common method (Ou *et al.*, 2001). The assay determines the scavenging capacity of a sample against oxygen radicals generated from the azo-radical initiator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). AAPH is thermally decomposed by heating the reaction mixture to 37°C, thus generating the oxygen radicals. By electron paramagnetic resonance (EPR) spin trapping studies, it has been shown that the oxygen radical generated by AAPH is an alkoxyl radical (Kraiev *et al.*, 1996; Sueishi *et al.*, 2010). Fluorescein (FL) (3',6'-dihydroxyspiro[2-benzofuran-3,9'-xanthene]-1-one) is used as a fluorescent probe and the fluorescence intensity (FI) of FL is continuously recorded for 35 min (Ou *et al.*, 2001). The FI of FL gradually decays owing to a reaction with the oxygen radical being generated. When an antioxidant is present, the fluorescence decay is slower than the decay in the absence of an antioxidant owing to scavenging of the oxygen radicals by the sample. The extent of this delay is represented by the area under the fluorescence decay curve (AUC). The net AUC, which is corrected by subtracting the AUC in the absence of the sample,

^{*}To whom correspondence should be addressed.

E-mail:krspmd@gmail.com

[†] Present address: 2-17-402, North-22 West-7, Kita-ku, Sapporo, Hokkaido 001-0022, Japan

indicates the radical scavenging activity of the sample. The radical scavenging activity of the sample is evaluated to be higher when the net AUC is increased (Cao *et al.*, 1993).

Interestingly, oxygen radicals can also be generated by photolysis of AAPH, i.e., through illumination with ultraviolet (UV) light (Kohri *et al.*, 2009; Sueishi *et al.*, 2010). The photolysis of AAPH efficiently generates a high quantity of oxygen radicals over a short time by illumination with UV light of adequate intensity, while no heating is required. Photolysis, therefore, allows the ORAC-FL assay to be implemented at room temperature, or even lower temperature. In addition, if the high concentration of oxygen radicals promotes fluorescence decay, the time required to monitor the fluorescence decay curve is reduced. It should be noted that the AAPH reaction mixture cannot be illuminated if the sample is present, as UV light may cause unexpected side-reactions. For instance, the flavonoids, a representative class of antioxidants originating from plants, are not only thought to act as radical scavengers, but also as sunscreens for sensitive biomolecules in plant cells because of their high UV absorbance. UV light can thus have various photochemical effects on flavonoids (Sisa *et al.*, 2010), and UV illumination should therefore be limited to AAPH to prevent any photodamage, though illumination of only AAPH in a mixed solution is impossible. Unfortunately, the addition of an illuminated AAPH solution (*i.e.*, after termination of illumination) to the FL-containing sample solution has no effect, because the generated radical rapidly disappears because of its extremely short lifetime.

Here, we found that radicals are also generated after termination of illumination, provided that the AAPH solution is appropriately cooled during illumination. This allows the implementation of a modified ORAC-FL assay, where radicals are generated by photolysis of AAPH. The purpose of this study was to evaluate the feasibility of an ORAC-FL assay, modified by employing the photolysis of AAPH. The experimental conditions for the modified ORAC-FL assay were determined and the feasibility of the modified assay was evaluated by actual sample measurements.

Materials and Methods

Materials AAPH, glutathione, caffeic acid, trolox, fluorescein sodium salt and sodium phosphate were purchased from Wako Pure Chemical Industries (Osaka, Japan). The spin trap compound 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline-N-oxide (CYPMPO) was obtained from Radical Research (Tokyo, Japan). All solutes were dissolved in 100 mM phosphate buffer (pH 7.4).

Instrumentation A UV illuminator (RUVF-203SR; Radical Research, Tokyo, Japan) was employed to generate

oxygen radicals from AAPH. The light source was a 200-W Xe arc lamp (San-ei Electronics, Osaka, Japan). The illuminator was equipped with a quartz fiber-optic guide and the guided light was emitted at the far end. The emission surface was a circle with a diameter of ca. 1 cm. The illuminator was equipped with a computer-controlled mechanical shutter, which controlled the illumination time. For EPR measurements, a JEOL RRX-1XS X-band ESR spectrometer (Tokyo, Japan) equipped with 100-kHz field modulation and WinRad operation software (Radical Research) was employed. The spectrometer settings were as follows: field modulation width, 0.1 mT; microwave power, 6 mW; field scan width/rate, ± 7.5 mT/2 min; and time constant, 0.1 s. A disposable borosilicate flat sample cell (Radical Research), which was loaded with sample liquid, was placed inside the EPR cavity and the EPR signal was recorded. Fluorescence measurements were performed using a SH-9000 microplate reader (Corona Electric, Ibaraki, Japan), which was modified to allow cuvette measurements. The excitation wavelength was 485 nm and the emission wavelength was 520 nm.

EPR spin trapping method The radicals generated from AAPH, after terminating illumination, were identified by EPR spin trapping method. A polypropylene tube containing 54 μ L of 0.5 mM AAPH solution was placed in an ice-salt bath ($< -10^{\circ}\text{C}$). The opening of the polypropylene tube was covered with the emission surface of the light guide and illuminated for 60 s. A 100 mM CYPMPO solution (6 μ L) was added to the AAPH solution 5 s after terminating illumination. The CYPMPO solution was added manually in synchrony with the elapsed time displayed on a digital timer. The mixture was loaded in the sample cell and the EPR signal was recorded. As a reference, the radicals produced during illumination were also identified. Here, the sample cell, loaded with 60 μ L of a solution containing 0.45 mM AAPH and 10 mM CYPMPO, was illuminated for 3 s inside the EPR cavity and the EPR signal was recorded immediately after terminating illumination.

Modified ORAC-FL assay The original ORAC-FL assay developed by Ou *et al.* was modified (Ou *et al.*, 2001), and the antioxidant capacity of caffeic acid and glutathione was evaluated using the modified assay. A cuvette containing 1200 μ L of 94.4 nM FL and either 120 μ L of 100 mM phosphate buffer (the blank) or 120 μ L of sample solution was placed in the fluorescence spectrometer. A polypropylene tube containing 450 μ L of a 15.9 mM AAPH solution was placed in an ice bath at 0°C . The opening of the polypropylene tube was covered with the emission surface of the light guide and illuminated for 1 s. The AAPH solution was transferred to the cuvette using a micropipette, 10 s after terminating illumination. The AAPH solution was added

Table 1. Summary of the calibration curve and modified ORAC-FL assay.

Run	Calibration Curve			Sample				
	Slope (s/ μ M)	Intercept (s)	R ² (a)	Name	Relative ORAC Value ^(b)	Slope (s/ μ M) ^(c)	Intercept (s) ^(c)	R ² (a)(c)
1	0.95	0.33	> 0.99	Caffeic acid	4.6 $\pm$ 0.7	3.4	7.6	0.95
2	0.68	4.5	> 0.99		4.7 $\pm$ 0.4	2.6	9.2	0.95
3	0.85	3.4	> 0.99		3.3 $\pm$ 0.2	2.4	7.4	0.99
4	0.68	4.3	0.97	Glutathione	0.56 $\pm$ 0.18	0.56	-3.3	0.93
5	0.91	3.6	> 0.99		0.49 $\pm$ 0.02	0.48	1.9	> 0.99
6	0.76	0.089	> 0.99		0.57 $\pm$ 0.04	0.51	-3.1	> 0.99
7	1.0	4.0	> 0.99					
8	1.2	7.0	0.98					
9	1.1	4.8	> 0.99					

^a Coefficient of determination.

^b Values are means and standard deviation of three concentrations. Values are given in moles of trolox equivalents per mole of sample.

^c Regression analysis of the relationship between three net AUCs and three concentrations of sample.

manually in synchrony with the elapsed time displayed on a digital timer. FI measurements were initiated 12 s before addition of an illuminated AAPH solution, and were conducted for 3 min with an interval of 3 s. The time of the first recording after AAPH addition was taken as 0 s. Each FI measurement was normalized to the intensity at 0 s. The AUC in the range 0 – 153 s was calculated using the trapezoidal rule. Five fluorescence decay curves were obtained for the blank and each concentration of sample, where the average of the corresponding five AUCs was employed for further analysis. Net AUC was obtained by subtracting the AUC from a blank measurement acquired at the same day. Regression analysis to estimate the relationship between trolox concentration and net AUC was based on three concentrations of trolox. The regression line was used as a calibration curve. The concentration of trolox giving a net AUC equal to that obtained for the sample was estimated by regression analysis. Net AUCs of the samples was obtained at three concentrations (caffeic acid: 5, 10, and 15 μ M; and glutathione: 30, 50, and 80 μ M). Relative ORAC values were expressed as equivalent moles of trolox per unit mole of sample. The average relative ORAC value at three sample concentrations was employed as the relative ORAC value of the sample. Each sample (caffeic acid or glutathione) was evaluated once a day for three days (Run 1 to Run 6) (Table 1). The calibration curve was created on the same day and the trolox concentrations were 30, 50 and 80 μ M in these Runs. The calibration curve was created once a day for three days (Run 7 to Run 9). The trolox concentrations were 10, 30 and 50 μ M in the three Runs.

For FI measurements other than the modified ORAC assay measurements, the same procedure was used. When the AAPH solution was illuminated at 5 or 10°C, a polypropylene tube was placed in a dry block thermostat.

Results

Figure 1a shows the EPR spectrum of the spin adduct upon addition of CYPMPO to an ice-cooled AAPH solution, 5 s after terminating illumination. The spectrum was identical to the spectrum obtained when a solution containing both CYPMPO and AAPH was illuminated (Fig. 1b). The peak-to-peak intensity in Fig. 1a was approximately a quarter of that observed in Fig. 1b. Figure 2 shows the fluorescence decay curves of FL upon addition of illuminated or non-illuminated AAPH solution. The relative FI displayed a steep decay after addition of illuminated AAPH solution. The time required to reach a relative FI below 10% was 30.8 ± 6.2 s,

Fig. 1. EPR spectra of the spin adduct of CYPMPO, prepared using different protocols.

(a) AAPH solution (0.5 mM in phosphate buffer (PB), 54 μ L) was illuminated for 60 s in an ice-salt bath, followed by CYPMPO addition (100 mM in PB, 6 μ L), 5 s after terminating illumination. The spectrum in Fig. 1 (a) was measured at quadruple the amplitude gain in Fig. 1 (b). The amplitude of the spectrum was reduced to a quarter of the original amplitude in Fig. 1 (a).

(b) Illumination of 0.45 mM AAPH and 10 mM CYPMPO (60 μ L in PB). Illumination time was 3 s.

Fig. 2. Fluorescence decay curves of FL solutions on addition of illuminated or non-illuminated AAPH solutions.

Illuminated AAPH (15.9 mM, 450 μ L in PB) was illuminated for 1 s at 0, 5 or 10°C, and was added to FL (85.8 nM, 1320 μ L in PB) at 10 s after terminating illumination. For non-illuminated samples, concentrations were: AAPH, 15.9 mM or 31.7 mM (450 μ L); and FL, 85.8 nM (1320 μ L).

while the final intensity was $0.46 \pm 0.002\%$ in the blank measurement of the modified assay ($n = 45$). On the other hand, when non-illuminated AAPH solution (15.9 mM) was added, the relative FI did not decay as steeply as for the illuminated AAPH and a gradual decay continued throughout the measurement. When non-illuminated AAPH solution of twice the concentration (31.7 mM) was added, the decay was similar. When the AAPH solution was illuminated at 5 and 10°C, the fluorescence decay was faster than at 0°C, and the final relative FI of the blank was $3.4 \pm 0.6\%$, and $5.9 \pm 1.2\%$, respectively ($n = 5$) (Fig. 2). When the illumination time of the AAPH solution was extended by 1 s, the final FI increased (Fig. 3). When an AAPH solution was illuminated for 1 s, an AAPH solution of twice the concentration did not decrease the final FI (Fig. 3).

The fluorescence decay curve of the blank and that in the presence of trolox are shown in Fig. 4. In the presence of trolox, the fluorescence decay curve reached a plateau, which was well above the final intensity of the blank. The level of the plateau increased with increasing concentrations of trolox.

The correlation between the concentration of trolox and the net AUC is plotted in Fig. 5 by employing the fluorescence decay curves displayed in Fig. 4, where the time range used to obtain the net AUC varied from 0 – 93 s to 0 – 333 s (*i.e.*, assays over several reaction times were simulated). The correlation depended on both the trolox concentration and the reaction time. The net AUC increased linearly for concentrations up to 50 μ M, independently of reaction time.

Fig. 3. Changes in final relative fluorescence intensity (FI) as a function of illumination time. FI was determined at 153 s after addition of AAPH. The depicted values are the average of 3 measurements. Error bars represent standard deviation. AAPH in phosphate buffer (15.9 or 31.7 mM, 450 μ L), illuminated on ice, was added to FL (85.8 nM, 1320 μ L in PB) at 10 s after terminating illumination.

Fig. 4. Fluorescence decay curves in the presence of various concentrations of trolox.

Trolox concentrations are as indicated. AAPH in phosphate buffer (15.9 mM, 450 μ L), illuminated for 1 s on ice, was added to a phosphate buffer solution containing FL (94.4 nM, 1200 μ L) and trolox (0 – 120 μ M, 120 μ L) at 10 s after terminating illumination. Fluorescence intensity was recorded for 348 s.

For reaction times longer than 153 s, at concentrations over 50 μ M, the net AUC also increased, and showed an upward shift as compared to the relationship found at lower concentrations. Conversely the net AUC showed a downward shift for reaction times of 93 s. The coefficient of determination showed a maximum value (0.9985) at 153 s in the 6 – 348 s reaction time range.

The results of the calibration curves and the modified as-

Fig. 5. Relationship of net AUC and trolox concentration.

Trolox concentrations are 0, 30, 50, 80, 100 and 120 μM . The time range used to calculate the net AUC varied from 0 – 93 s to 0 – 333 s.

say are shown in Table 1. Except for the fourth and eighth runs, the coefficient of determination of the calibration curve was over 0.99, and exhibited a good linearity. The linearity between the net AUC and the concentration was also evaluated for caffeic acid and glutathione. For both samples, the fluorescence decay curve reached a plateau (Fig. 6). The coefficient of determination of the samples was lower than the lowest value (0.97) for the trolox calibration curve for three runs (Table 1). The relative ORAC values of caffeic acid and glutathione were 4.2 ± 0.6 (15%), and 0.54 ± 0.04 (6.6%), respectively ($n = 3$, value within the parentheses is the relative standard deviation). The relative ORAC values for caffeic acid and glutathione reported by Ou *et al.* were 4.37 ± 0.24 , 0.62 ± 0.02 , respectively (Ou *et al.*, 2001). Our results differed by -0.17 (relative difference: -4.5%) and -0.08 (relative difference: -12.5%), respectively.

Discussion

By photolysis of AAPH, the time required for monitoring the fluorescence decay curve was decreased, and the oxygen radical activity of the ORAC assay was evaluated at room temperature.

Interestingly, after terminating illumination, radicals could still be detected in solution (Figs. 1a and 1b). As the fluorescence intensity did not steeply decay with addition of non-illuminated AAPH solution at two-fold concentration, radical generation appears to be induced by illumination, and not via thermal decomposition (Fig. 2). The mechanism of radical generation is thought to be as follows. Several azoalkanes in which two alkyl groups are bonded to the azo group

Fig. 6. Fluorescence decay curves in the presence of trolox, caffeic acid and glutathione.

form stable trans-configurations. By absorption of UV light, the trans-configuration is transformed into unstable cis-configuration. The unstable cis-isomer then decomposes into two alkyl radicals and a nitrogen molecule, where the stability of the cis-isomer depends on the nature of the alkyl groups (Engel, 1980). Below -50°C *trans*-azoisobutane, for instance, isomerizes to the cis-isomer upon illumination with light of wavelengths lower than 400 nm. The cis-isomer then rapidly decomposes, accompanied by evolution of nitrogen, upon warming to 25°C (Mill and Stringham, 1969). Indeed, AAPH also decomposes rapidly, via isomerization of the stable trans-isomer to the unstable cis-isomer by absorption of UV light. At room temperature, this unstable cis-isomer decomposes rapidly. The decomposition rate is expected to decrease by cooling on ice, which allows the remaining cis-isomer to generate radicals, even after terminating illumination. Indeed, this reasoning is supported by the present experiments. When the AAPH solution was illuminated at temperatures higher than 0°C , the speed of the fluorescence decay and the final value of the relative FI of the blank increased (Fig. 2). The increase of the decay speed can be explained by the increase in the decomposition rate of the cis-isomer in the assay mixture, which was added by AAPH solutions kept at temperatures higher than 0°C . The increase in the final fluorescence intensity can be explained by the more rapid decomposition rate of the cis-isomer at higher temperatures during illumination, as compared to that observed at the temperature of the ice bath used in the modified ORAC-FL assay. To fully confirm this mechanism, direct observation of the cis-isomer is required. Details on the behavior of the cis-isomer would

allow the evidence-based optimization of the parameters used to generate the oxygen radical, such as illumination power and time, cooling temperature and AAPH concentration. In this study, the instability of the cis-isomer may have partly accounted for the experimental variations observed. For instance, the time required to transfer the AAPH solution varied slightly from trial to trial because of the uncertainties associated with its manual addition. This slight variation in the processing time could have led to significant variations in the concentration of remaining cis-isomer as a result of its rapid decomposition rate. As a consequence, the concentration of cis-isomer added may have varied. It is also possible that the decomposition rate of the cis-isomer is accelerated at room temperature. These issues could be addressed by mechanical transfer of the AAPH solution or by cooling the AAPH solution during transportation, which would prevent the decomposition rate from increasing.

It was observed that, in the presence of trolox, the fluorescence decay curve reached a plateau, which was well above the final intensity of the blank (Fig. 4), consequently giving rise to a relatively complex relationship between net AUC and trolox concentration (Fig. 5). The generation of oxygen radicals apparently stopped when the fluorescence decay curve reached a plateau, which is different from the original ORAC-FL assay, where the oxygen radical is generated continuously. The net AUC obtained was overestimated because of the area under the curve in the plateau region, as compared to the continuous decay curve. As a result, the conditions (*i.e.*, time range of the fluorescence decay curve and trolox concentrations) for which a linear relationship existed between the net AUCs and trolox concentrations needed to be investigated. Irrespective of the time range, a linear relationship between net AUC and trolox concentration was observed at low trolox concentrations, owing to the low level of the plateau (Fig. 5, 0–50 μM). As the concentration increased, the overestimation of the net AUC became more significant, owing to the contribution of the plateau region (Fig. 5, 0–213 s, 0–273 s, and 0–333 s range, 80–120 μM). Conversely, the net AUC was underestimated in the 0–93 s range, owing to the short time range. In the 0–153 s range (used in this study) the two opposite trends balanced, and a linear relationship was observed up to 120 μM . As the net AUC was formed mainly in the initial recording period for trolox, this time range was found to be effective for creating a calibration curve. In cases where the fluorescence decay curve does not display the same characteristics, this approach may not always be effective, and the linearity observed for caffeic acid and glutathione is usually lower than that of trolox (Table 1). As compared to trolox, the contribution to net AUC after longer recording periods was significant

for caffeic acid and glutathione (Fig. 6). The relative ORAC values were lower than those of the original ORAC-FL assay in caffeic acid, and glutathione. A plateau with higher levels was observed for the time range. As the net AUC was overestimated by the plateau, the lower relative ORAC values were not caused by the plateau. Fluorescence measurement and analysis in the modified ORAC-FL assay are almost the same as in the original assay. The difference is therefore thought to originate from the high concentration of oxygen radicals, or the transient temperature inhomogeneity of the assay mixture (see below). Irrespective, it is recommended to keep the plateau region of the fluorescence curve, which was not originally considered in the ORAC-FL assay, as low as possible. The use of a low concentration of antioxidant is therefore recommended in the modified assay. Alternatively, any increase in the concentration of generated radicals could decrease the plateau level. Here, cooling the AAPH solution during transferring or shortening the transfer time could be of utility to limit the loss of cis-isomer during transportation of AAPH solution. Other possibilities include decreasing the temperature used to cool the AAPH solution or increasing the concentration of generated cis-isomer, which could be effected by increasing the illumination power.

Because the fluorescence spectrometer employed in this study was not equipped with a cooling device, the measurement solution in the cuvette was not cooled. Therefore, transient temperature inhomogeneities could have occurred in the assay mixture upon addition of the cooled AAPH solution. As a result, the radical scavenging reactions proceeded at a non-specified temperature in this study, which could have accounted for the variations in the results. For evaluation of radical scavenging activity, such experimental conditions should preferably be avoided. When both solutions (and assay mixture during assay) are cooled at the same low temperature, the antioxidant activity at the temperature under consideration can be evaluated, allowing the evaluation of the radical scavenging activity at temperatures lower than room temperature. This may also improve experimental conditions, as the evaluation of antioxidant capacities at low temperature could provide relevant information. Yamamoto *et al.* (1999), for instance, found an isomer of α -tocomonoenol in eggs of chum salmon, which was designated marine-derived tocopherol (MDT). The peroxy radical-scavenging activity of MDT and α -tocopherol were compared at 37°C, and were found to be equivalent. In contrast, at 0°C, MDT inhibited peroxidation more effectively than α -tocopherol (Yamamoto *et al.*, 2001). These results were suggested to result from adaptation to the high oxidative stress levels in cold sea waters, as the MDT content (as part of total tocopherol derivatives) in fish inhabiting cold sea waters was higher than that in fish

inhabiting tropical waters. When the temperature at which antioxidant activity is evaluated is higher than the temperature at which the antioxidant is supposed to function, it is possible that its intrinsic activity is not properly evaluated. Samples prepared from organisms inhabiting cold environments, such as MDT and antioxidant additives employed at low temperature in food or in industrial products, fall under this condition. The application range of the modified ORAC assay is, therefore, expected to be broadened by its application at low temperature.

The period required is comparable to that obtained with other methods (*i.e.*, by FRAP (ferric reducing ability of plasma): 4 min, and TEAC (trolox equivalent antioxidant activity): 4 – 6 min), and is shorter than the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (20 min) (Benzie *et al.*, 1996; Re *et al.*, 1999; Yamaguchi *et al.*, 1998). The decrease in the monitoring time has some important implications. One application, for instance, is to evaluate temporal changes in antioxidant activity by sequentially withdrawing portions from a sample, at even intervals. Importantly, this interval is much shorter than the period required to conduct the original ORAC-FL assay. Although it is, in principle, possible to simultaneously measure many previously stored samples, the modified ORAC-FL does not require such a strategy. The modified ORAC-FL assay is thus suitable for assessing thermally labile samples, which may degenerate during the long measurements required for the original ORAC-FL assay. It should be noted that the original ORAC-FL assay is usually implemented on a microplate reader, which allows the automated measurement of many samples at once (Huang *et al.*, 2002). Ninety-six samples can be evaluated by using a 96-well microplate for 35 min in the original ORAC-FL assay. When 96 samples are evaluated using the modified ORAC-FL assay, more than 4 h of measurement time is required. The advantage of the decreased measurement time for the modified ORAC-FL is in this case canceled out by the successive measurement of multiple samples. However, equipping a microplate reader with the instrumentation required for the modified ORAC-FL assay, such as an illumination system for the photolysis, would shorten the assay time, regardless of the number of samples.

To date, the modified ORAC-FL assay suffers from some minor shortcomings. First, the fluorescence decay curve was found to reach a plateau, which is not in accordance with the original ORAC-FL assay. Although the continuous generation of the oxygen radical, as in the original assay, is preferred, the increased concentration of the generated oxygen radical and the low concentration of the antioxidant contribute to a decrease in the plateau level in the modified assay. A second shortcoming is that the radical scavenging

reaction proceeded at an unknown temperature. The cooling of the AAPH solution is essential for the radical generation of the modified assay and the solutions should be maintained at the same temperature. It is possible that radical generation is extended by the decrease in the decomposition rate of the cis-isomer at low temperatures, which may in turn decrease the plateau level. A third issue is that the mechanism of radical generation has not been fully elucidated. As the oxygen radicals are generated only after illumination, this does not necessarily present a practical problem, although it should be noted that mechanistic elucidation would be useful for interpretation of the results and any future improvements.

In conclusion, the feasibility of the modified ORAC-FL assay should be evaluated for more samples so as to compare it with the original ORAC-FL assay, while the various shortcomings should be addressed.

Acknowledgements This work was supported by a grant from Sapporo Biocluster “Bio-S”, the Knowledge Cluster Initiative of the Ministry of Education, Sports, Science, and Technology (MEXT).

References

- Benzie, I.F.F. and Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of “Antioxidant Power”: The FRAP assay. *Anal. Biochem.*, **239**, 70-76.
- Cao, G., Alessio, H.M. and Cutler, R.G. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radic. Biol. Med.*, **14**, 303-311.
- Engel, P.S. (1980). Mechanism of the thermal and photochemical decomposition of azoalkanes. *Chem. Rev.*, **80**, 99-150.
- Fernández-Sánchez, A., Madrigal-Santillán, E., Bautista, M., Esquivel-Soto, J., Morales-González, Á., Esquivel-Chirino, C., Durante-Montiel, I., Sánchez-Rivera, G., Valadez-Vega, C. and Morales-González, J.A. (2011). Inflammation, Oxidative Stress, and Obesity. *Int. J. Mol. Sci.*, **12**, 3117-3132.
- Guo, C., Cao, G., Sofic, E. and Prior, R.L. (1997). High-performance liquid chromatography coupled with coulometric array detection of electroactive components in fruits and vegetables: Relationship to oxygen radical absorbance capacity. *J. Agric. Food Chem.*, **45**, 1787-1796.
- Ha, H-L., Shin, H-J., Feitelson, M.A. and Yu, D-Y. (2010). Oxidative stress and antioxidants in hepatic pathogenesis. *World J. Gastroenterol.*, **16**, 6035-6043.
- Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J.A. and Prior, R.L. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.*, **50**, 4437-4444.
- Huang, D., Ou, B. and Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.*, **53**, 1841-1856.

- Karadag, A., Ozcelik, B. and Saner, S. (2009). Review of methods to determine antioxidant capacities. *Food Anal. Methods*, **2**, 41-60.
- Kohri, S., Fujii, H., Oowada, S., Endoh, N., Sueishi, Y., Kusakabe, M., Shimmei, M. and Kotake, Y. (2009). An oxygen radical absorbance capacity-like assay that directly quantifies the antioxidant's scavenging capacity against AAPH-derived free radicals. *Anal. Biochem.*, **386**, 167-171.
- Krainev, A.G. and Bigelow, D.J. (1996). Comparison of 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) as free radical initiators: A spin-trapping study. *J. Chem. Soc., Perkin Trans.*, **2**, 747-754.
- Lonkar, P. and Dedon, P.C. (2011). Reactive species and DNA damage in chronic inflammation: Reconciling chemical mechanisms and biological fates. *Int. J. Cancer*, **128**, 1999-2009.
- Lordan, S., Ross, R.P. and Stanton, C. (2011). Marine bioactives as functional food ingredients: Potential to reduce the incidence of chronic diseases. *Mar. Drugs*, **9**, 1056-1100.
- Mill, T. and Stringham, R.S. (1969). Photoisomerization of azoalkanes. *Tetrahedron Lett.*, **10**, 1853-1856.
- Ou, B., Hampsch-Woodill, M. and Prior, R.L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.*, **49**, 4619-4626.
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.*, **26**, 1231-1237.
- Sisa, M., Bonnet, S.L., Ferreira, D. and Van der Westhuizen, J.H. (2010). Photochemistry of flavonoids. *Molecules*, **15**, 5196-5245.
- Sommerburg, O., Keunen, J.E.E., Bird, A.C. and van Kuijk, F.J.G.M. (1998). Fruits and vegetables that are sources for lutein and zeaxanthin: The macular pigment in human eyes. *Br. J. Ophthalmol.*, **82**, 907-910.
- Sueishi, Y., Yoshioka, D., Oowada, S., Endoh, N., Kohri, S., Fujii, H., Shimmei, M. and Kotake, Y. (2010). Is the oxygen radical absorbance capacity (ORAC) method a peroxyl-radical scavenging assay?. *Z. Phys. Chem.*, **224**, 921-928.
- Traka, M.H. and Mithen, R.F. (2011). Plant science and human nutrition: Challenges in assessing health-promoting properties of phytochemicals. *Plant Cell*, **23**, 2483-2497.
- Yamaguchi, T., Takamura, H., Matoba, T. and Terao, J. (1998). HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Biosci. Biotechnol. Biochem.*, **62**, 1201-1204.
- Yamamoto, Y., Maita, N., Fujisawa, A., Takashima, J., Ishii, Y. and Dunlap, W.C. (1999). A new vitamin E (α -tocomonoenol) from eggs of the pacific salmon *Oncorhynchus keta*. *J. Nat. Prod.*, **62**, 1685-1687.
- Yamamoto, Y., Fujisawa, A., Hara, A. and Dunlap, W.C. (2001). An unusual vitamin E constituent (α -tocomonoenol) provides enhanced antioxidant protection in marine organisms adapted to cold-water environments. *Proc. Natl. Acad. Sci. USA*, **98**, 13144-13148.

URL Cited

- i) http://www.who.int/topics/chronic_diseases/en/ (Aug 21, 2012)